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Post-transcriptional effects of phorbol 12-myristate 13-acetate on transcriptome of U937 cells

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Abstract To identify post-transcriptionally modulated genes at the translational level by phorbol 12-myristate 13-acetate (PMA), we investigated mRNA profiles in the polysomal and the cytoplasmic fractions of U937 cells before and after PMA stimulation using microarrays with 15 017 oligonucleotide probes. Global comparison of the profiles showed that the cytoplasmic distribution of mRNAs was considerably modulated upon PMA stimulation. The results also indicate that PMA post-transcriptionally regulated at least 0.7% of detectable genes in U937 cells. Thus, besides transcriptional modulation by PMA, changes in the translational state of transcripts seem to play a critical role in PMA-induced differentiation of U937 cells.
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Keywords: Phorbol 12-myristate 13-acetate; Transcriptome; Post-transcriptional regulation; Polysome; Cytoplasm; Macrophage

1. Introduction

Exudate macrophages play pivotal roles in inflammatory and immune responses. This type of cell is considered to originate from circulating monocytes, and to differentiate in response to several stimulants such as cytokines and bacterial components. To understand the molecular mechanism underlying the macrophage maturation, several immature monocytic cell lines such as U937 and HL60 are widely used as models [1,2]. Stimulation of the cells with phorbol 12-myristate 13-acetate (PMA), a canonical protein kinase C activator, induces maturation resulting in several macrophage-like phenotypes, such as cell body expansion, termination of proliferation, and antigen presentation [1]. So far, a number of genes and proteins have been identified as being modulated by PMA-induced differentiation [2–4]. However, because it is well known that the correlation of the mRNA levels with the protein levels is not necessarily high, more detailed information is needed to describe the molecular events taking place in the cells on a molecular basis.

Microarray analysis is a powerful approach to obtain expression data of tens of thousands of genes in a single experiment and, for convenience, is frequently conducted using total cellular RNA or total cytoplasmic RNA. However, in this study, we intended to use microarray analysis to detect post-transcriptional controls of gene expression exerted by PMA. To achieve this, we compared the mRNA levels in the polysomal and the cytoplasmic fractions by microarray experiments. Since polysomes are the subcellular fraction of ribosomes actively engaged in protein synthesis, this approach enabled us to monitor the translational states of more than 10 000 mRNA species before and after the PMA stimulation [5,6]. Using U937 cells, we successfully identified 105 genes which were under post-transcriptional control in the PMA-induced differentiation.

2. Materials and methods

2.1. Cells and RNA preparation

Human histomonocytic cell line U937 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI1640 supplemented with 10% fetal calf serum. The cells were differentiated by treatment with PMA (32 nM; Sigma, St. Louis, MI, USA) for 48 h. Cytoplasmic RNA was prepared as previously described [6]. For polysomal RNA isolation, cytoplasmic RNA was further applied to fractionation using a 15–40% sucrose gradient as described [6]. Aliquots of each fraction were electrophoresed on 1.2% agarose/formaldehyde gels, transferred, fixed onto a nylon membrane, and stained with methylene blue. The membranes were hybridized with an α ^{32}P-dCTP labeled β-actin cDNA as a reference.

2.2. Microarray analysis

cDNA synthesis and subsequent aRNA amplification were conducted using a kit purchased from Ambion (Austin, TX, USA). The amplified aRNA was directly labeled with either Cy3 or Cy5 by Micromax ASAP RNA labeling kit (Perkin Elmer; Wellesley, MA, USA). Five hundred ng each of Cy3- and Cy5-labeled aRNAs were mixed in a hybridization cocktail (Agilent; Santa Clara, CA, USA), and then hybridized with a human oligo 1A microarray slide (Agilent) for 16 h. After washing as described in the manufacturer's instructions, the arrays were scanned using a microarray scanner (Agilent). Data mining and LOWESS normalization were performed using a feature extraction program (Agilent). Both per chip and per spot normalizations were conducted using a median correction program in the Genespring software package (Silicon Genetics; Redwood City, CA, USA). One comparison between two groups was conducted using a duplicate

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Abbreviations: ARE, adenylate/uridylate-rich element; PMA, phorbol 12-myristate 13-acetate; UTR, untranslated region

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array, each of which consisted of the mean of the dye-swapped array data. In this study, the difference of the microarray data was recognized as "significant" when it satisfied all the following three criteria: (1) the average ratio of expression levels was more than 2-fold, (2) *P*-value provided by the feature extraction was representatively less than 0.01, and (3) the ANOVA program in Genespring at default setting indicated a significant difference ($P < 0.01$) between the samples. Gene tree clustering was based on the results of a standard correlation program packaged in Genespring.

2.3. Real-time PCR analysis

Isolation of mRNA from total RNA was performed by the μMACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). mRNA contents of each sample were determined by the Ribo Green RNA quantification kit (Invitrogen). Pre-designed and gene-specific Taqman probe and primer sets were purchased from Applied Biosystems (Foster City, CA, USA). Real time PCR was performed using the ABI 7000 analyzer (Applied Biosystems) according to the manufacturer's directions. All values were expressed as means±S.D. Statistical comparisons were made by ANOVA, followed by Tukey's Honestly Significant Difference test.

3. Results

3.1. Global comparison of transcriptomes between the cytoplasmic and the polysomal fractions of U937 cells before and after PMA stimulation

After 48 h stimulation with 32 nM PMA, U937 cells differentiated into macrophage-like cells: the cells became adhesive and expanded (Fig. 1A). We isolated total cytoplasmic RNAs from the cells before and after PMA stimulation, and subsequently subjected them to the microarray analysis. Among 15 017 oligonucleotides probes, 303 probes detected differentially expressed transcripts: 163 of them were upregulated, while 140 were downregulated (Fig. 2A). The list included well-known differentiation markers of U937 cells, such as matrix metalloproteinase 9 and osteopontin. All the raw data described in this study were deposited to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE1783.

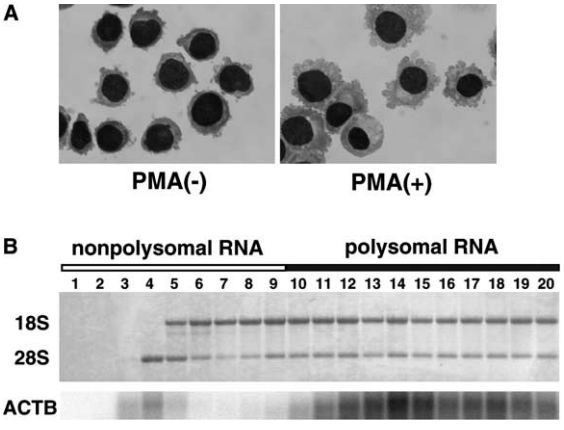

Fig. 1. Preparation of U937 cells and their polysomal RNA. (A) Intact and PMA-stimulated (32 nM, 48 h) cells were stained with Giemsa solution. (B) Representative polysome profile of U937 cells. Total RNA (1 μg) was extracted from each of the 20 sucrose gradient fractions, transferred onto a nylon membrane, and stained with methylene blue. Fractions 10–20 correspond to polysomal RNA. The membrane was hybridized with a β-actin (ACTB) as a reference.

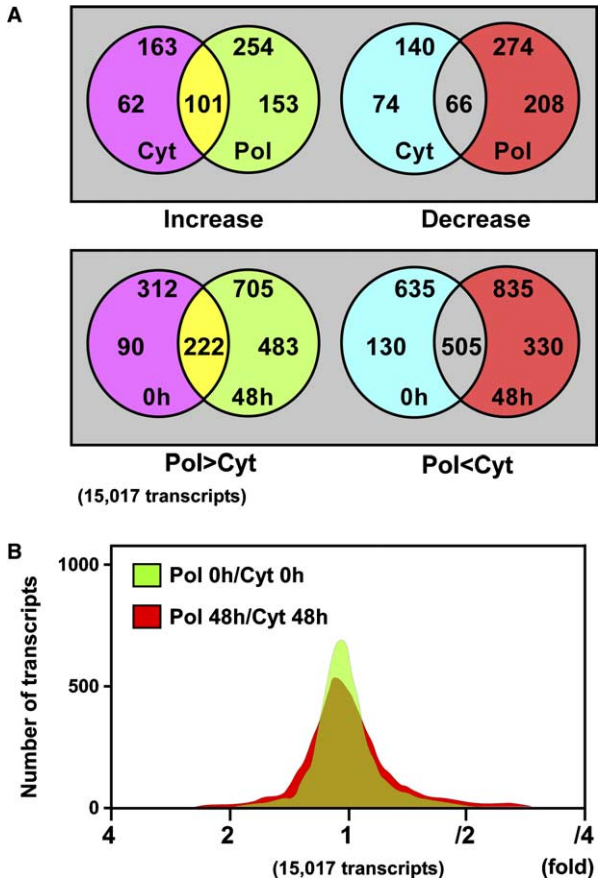

Fig. 2. Global comparison between cytoplasmic and polysomal expression profiles. (A) Venn diagram indicating the number of increased and decreased transcripts in the cytoplasm and polysome by the PMA stimulation (upper panel), and the number of transcripts differentially distributed in the fractions (lower panel). (B) Comparison of transcript distribution between 0 h (green) and 48 h (red) after PMA stimulation. Pol, polysomal RNA; Cyt, cytoplasmic RNA.

To profile the transcriptome consisting of mRNAs engaging in protein synthesis, we next purified polysomal RNAs by sucrose density gradient centrifugation and subjected them to microarray analysis (Fig. 1B). In this fraction, a larger number of transcripts were influenced by PMA stimulation as in the total cytoplasmic fraction: 254 and 274 genes were up and downregulated, respectively, with statistical confidence (Fig. 2A). Interestingly, the Venn diagrams shown in Fig. 2A indicated that 60.2% of the upregulated and 75.9% of the downregulated mRNA levels by the PMA stimulation were modulated specifically in the polysomal fraction, while 38.0% of the increased and 52.9% of the decreased mRNA levels in the cytoplasm were not affected in the polysomal fraction. The mRNA levels in the cytoplasmic and the polysomal fractions and their changes in response to the PMA-induced differentiation were further examined. In the immature cells, 947 transcripts were unevenly located in either of the polysomal or the total cytoplasmic fraction (Fig. 2A). Furthermore, a larger number of transcripts (1540) changed their localization pattern between the fractions after the differentiation. Consequently, the ratios of the mRNA levels in the polysomal and the cytoplasmic fractions, hereafter termed the P/C ratio, of the differentiated cells varied more widely than those in the immature cells (Fig. 2B).

These results indicate that a considerable portion of transcripts was regulated post-transcriptionally and that the post-transcriptional regulation was more profound in the differentiated cells than in the immature cells.

3.2. Identification of transcripts exhibiting re-allocation between subcellular fractions after PMA stimulation

To further identify transcripts altering their cytoplasmic distribution by PMA stimulation, we extracted genes of which the mRNA levels were affected only in a single fraction and accompanied by statistically confident changes in their P/C ratio. One hundred and five transcripts satisfied these criteria and were subsequently subjected to gene tree clustering analysis based on a standard correlation method (Fig. 3). Among them, 66 transcripts exhibited significant changes in the mRNA levels only in the polysomal fraction after the stimulation, resulting in biased distribution in the differentiated cells. In contrast, nine transcripts were more abundant in the polysomal fraction in the immature cells, while this maldistribution was overcome in the mature cells by polysome-specific decrement. PMA also evoked total cytoplasm-specific changes in the mRNA levels of 30 genes also with changes in the P/C ratio.

3.3. Confirmation of the microarray data by real time PCR analysis

To confirm the microarray data, we further analyzed mRNA levels of some genes by real time PCR. We examined eight genes for which the mRNA levels were altered only in the polysomal fraction; they included four macrophage-related genes [C-type lectin superfamily member 5 (CLECSF5), early growth response (EGR) 1, αV-integrin (ITGAV) and a disintegrin and metalloproteinase domain (ADAM) 10] and four randomly selected genes [mannosyl (α-1,3-)-glycoprotein β-1,2-*N*-acetylglucosaminyl transferase (MGAT1), caspase recruitment domain family member (CARD) 6, FLJ20303, and basic leucine zipper transcription factor, ATF-like (BATF)]. As shown in Fig. 4, the mRNA levels encoding CLECSF5, MGAT1, CARD6 and FLJ20303 protein distinctively increased in the polysomal fractions after the PMA stimulation. Although PMA also elevated their mRNA levels of these genes in the total cytoplasmic fraction, they were not statistically significant. Likewise, the mRNA levels of EGR1, ITGAV and ADAM10 were elevated more dramatically in the polysomal fraction compared with the cytoplasmic one. Since the elevation of the mRNA levels of these genes in the cytoplasmic fraction was statistically significant, these genes were considered to be controlled post-transcriptionally as well as transcriptionally. Conversely, PMA attenuated entry of BATF mRNA into the polysomal fraction after the stimulation.

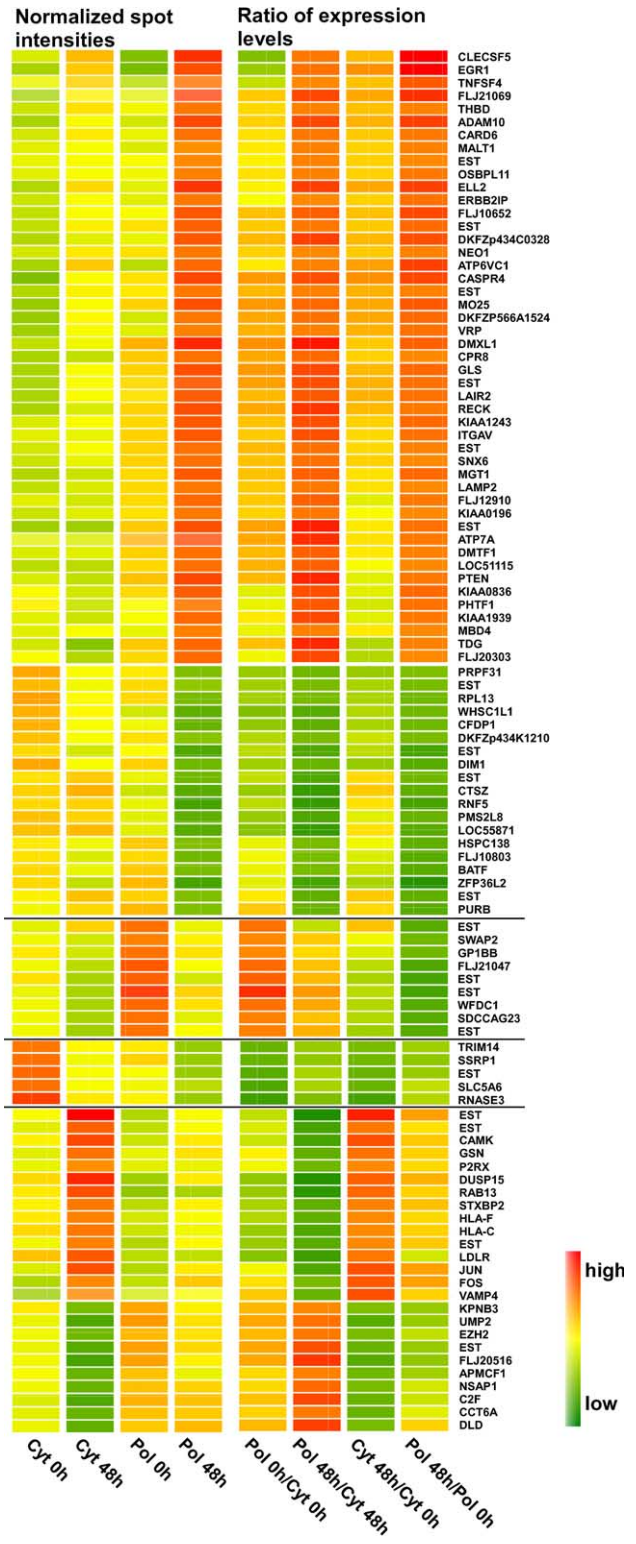

Fig. 3. Transcripts post-transcriptionally regulated during differentiation. Gene tree involving 105 genes for which the expression levels were altered with PMA in a single fraction, concurrently with changes in the polysomal/cytoplasmic ratio. Normalized expression values (left) and ratio of expression levels (right) are shown here. Pol, polysomal RNA; Cyt, cytoplasmic RNA.

4. Discussion

The results in this study showed that mRNAs in the polysomal fraction responded to the PMA stimulation differently from those in the cytoplasmic fraction. Since polysomal mRNAs are the fraction of mRNAs engaging in protein synthesis, we predicted that the mRNA profiling data originating from the polysomal fraction more faithfully reflected protein profiles at synthesis than those derived from the total cytoplasmic mRNAs [5,6]. Thus, the data obtained by the microarray experiments using subcellularly fractionated mRNAs may enable us to bridge the gap between the transcriptome and the

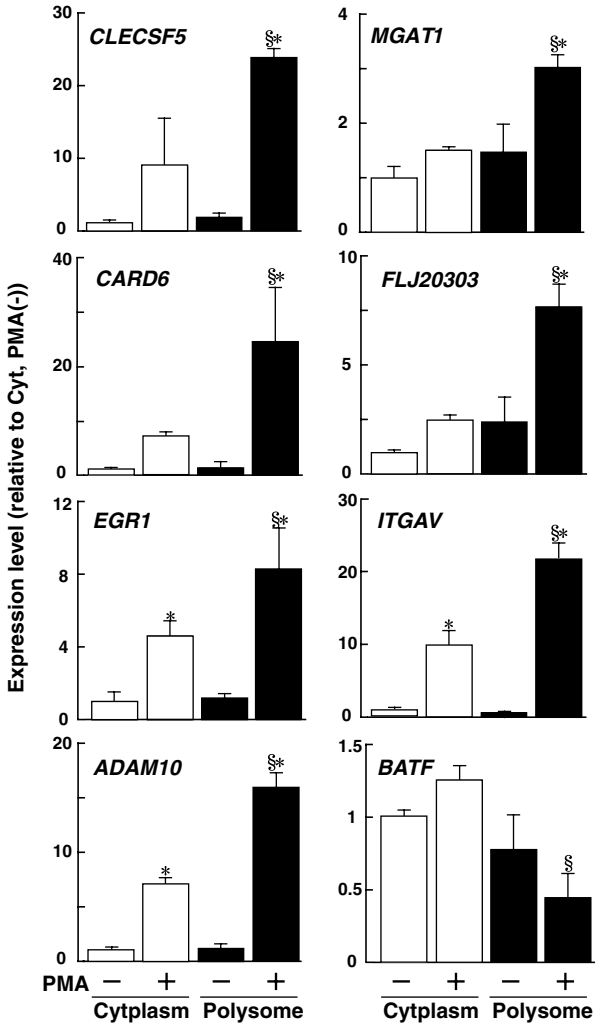

Fig. 4. Real time PCR analysis using the total cytoplasmic and polysomal RNA. mRNA levels of each transcript are expressed as relative to the average of total cytoplasm in the intact cells. Values are means ± S.D. for three independent samples. §$P < 0.05$ and *$P < 0.05$ are compared with the cytoplasmic RNA and intact cell values.

proteome, which is of considerable importance in functional genomic investigations. Furthermore, the comparative analysis of the data of mRNA profiles in the polysomal and the cytoplasmic fractions must be informative because this allows us to discriminate post-transcriptionally induced change from transcriptionally induced change, as demonstrated in this study. In fact, we found that at least 0.7% (105/15 017) of cytoplasmic mRNA species significantly changed subcellular localization during the differentiation of U937 cells. This was the first time this had been done. Moreover, the results in this study enabled us to classify genes according to the stage at which their gene expression was regulated in response to the PMA stimulation. The accumulation of this type of data, using different cell types and different stimuli, will present new opportunities to obtain a more comprehensive understanding of gene regulation networks in mammalian cells.

In agreement with array experiments, real time PCR analyses verified that some macrophage-related transcripts were actually regulated in the polysomal fraction. CLECSF5 is abundant on the surface of differentiated myeloid cells [7]. Association of this with a protein kinase DAP12 caused calcium mobilization in macrophages [7]. ADAM10 can convert precursors of some cytokines, such as TNF and CX3CL1, into their active forms [8,9]. EGR1, a zinc finger protein, is an essential regulator of myeloid cell differentiation because application of its antisense oligonucleotide prevented differentiation of U937 and HL60 cells [10]. ITGAV is a fibronectin receptor and tethers mature macrophages to lesions [11]. Until now, some reports demonstrated that EGR1 and ITGAV mRNAs were upregulated by PMA stimulation in macrophages at total RNA levels [2,11]. The present results demonstrate that post-transcriptional as well as transcriptional regulatory mechanisms underlay induction of these critical components in mature macrophages.

After transcription, several mRNAs are transported into specific regions, such as the cell periphery and perinuclear cytoplasm, and then eventually subjected to translation. In Aplysia neural cells, eukaryotic translation elongation factor 1α mRNA was conveyed into neurites by axonal transport, being essential for maintaining newly grown synapses [12]. Translocation of mRNAs into translation sites is considered to be mediated by cytoskeletal elements, such as microfilaments and microtubules [13]. Moreover, a significant portion of polysomes attached to the cytoskeleton as well as the membrane [13,14]. Thus, it may be argued that the cytoskeleton is one of the pivotal post-transcriptional regulators in cells. Recently, Brock et al. [15] reported that PMA-induction resulted in the reorganization of the cytoskeleton and the association of transcripts with the cytoskeleton in HL60 cells: 18 out of 649 transcripts associated with cytoskeleton in the differentiated cells, whereas only 6 transcripts in the immature cells. Our results also indicate that a considerable number of mRNA species were re-allocated in subcellular fractions after the PMA-stimulation and it is also very likely that cytoskeleton reorganization must occur in differentiated U937 cells, since the cytoplasmic space was clearly expanded by the PMA stimulation. In future, this may lead to the elucidation of an unexplored role of the cytoskeleton as a scaffold of protein synthesis.

In addition to cytoskeletal architecture, post-transcriptional control can be mediated by *trans*-acting proteins [16]. These proteins generally bind structural elements in the untranslated region (UTR) of the target mRNA. In particular, the adenylate/uridylate-rich element (ARE) in the 3′UTR is the best documented of such elements in eukaryotes [17,18]. ARE is frequently found in mRNAs of oncogenes and cytokines, and affects their localization, efficacy of translation, and stability [17,18]. Moreover, it is also known that PMA destabilizes several transcripts in an ARE-directed manner, suggesting that ARE is one of the major target sites of PMA-induced responses [17]. In fact, our preliminary in silico search actually detected a typical ARE in some of the 105 transcripts identified to be post-transcriptionally regulated in this study. Further studies are required to clarify whether ARE and its binding proteins are responsible for the post-transcriptional response to PMA stimulation.

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